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Microarrays of cells expressing defined cDNAs

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Genome and expressed sequence tag projects are rapidly cataloguing and cloning the genes of higher organisms, including humans. An emerging challenge is to rapidly uncover the functions of genes and to identify gene products with desired properties. We have developed a microarray-driven gene expression system for the functional analysis of many gene products in parallel. Mammalian cells are cultured on a glass slide printed in defined locations with different DNAs. Cells growing on the printed areas take up the DNA, creating spots of localized transfection within a lawn of non-transfected cells. By printing sets of complementary DNAs cloned in expression vectors, we make microarrays whose features are clusters of live cells that express a defined cDNA at each location. Here we demonstrate two uses for our approach: as an alternative to protein microarrays for the identification of drug targets, and as an expression cloning system for the discovery of gene products that alter cellular physiology. By screening transfected cell microarrays expressing 192 different cDNAs, we identified proteins involved in tyrosine kinase signalling, apoptosis and cell adhesion, and with distinct subcellular distributions.

The growing collection of gene sequences and cloned cDNAs demands the development of systematic and high-throughput approaches to characterizing the gene products. Strategies comparable to DNA microarrays for transcriptional profiling[1,2] and to yeast two-hybrid arrays for determining protein–protein interactions[3] do not exist to analyse the function, within mammalian cells, of large sets of genes. At present, *in vivo* gene analysis can be done—on a gene-by-gene scale—by expressing a DNA construct within cells that directs the overproduction of a gene product or inhibits its synthesis or function. The effects on cellular physiology of altering the level of a gene product is then detected using a variety of functional assays. We describe a strategy for the high-throughput analysis of gene function in mammalian cells. We have developed a system suitable for rapidly screening large sets of cDNAs or DNA constructs for those genes encoding desired products or causing cellular phenotypes of interest. Using slides printed with sets of cDNAs in expression vectors, we create living microarrays of cell clusters expressing the gene products. The cell clusters can be screened for any property detectable on a surface and the identity of the responsible cDNA determined from the coordinates of the cell cluster with a phenotype of interest.

To create these microarrays, we simultaneously transfect distinct and defined areas of a lawn of cells with different plasmid DNAs (Fig. 1a). This is done without the use of individual wells to sequester the DNAs. Nanolitre volumes of plasmid DNA in an aqueous gelatin solution are printed on a glass slide using a robotic arrayer. After drying, the DNA spots are briefly exposed to a lipid transfection reagent; the slide is then placed in a culture dish and covered with adherent mammalian cells in medium. The cells growing on the DNA and gelatin spots express the DNA and divide 2–3 times in the process of creating a microarray with features consisting of clusters of transfected cells (which we call 'transfected cell microarrays'). We call the method to make the arrays 'reverse transfection' because, compared with conventional transfection, we have reversed the order of addition of DNA and adherent cells. For detailed protocols of this and alternative methods see http://staffa.wi.mit.edu/sabatini_public/reverse_transfection.htm.

To illustrate the method, we printed an array with elements containing an expression construct for the green fluorescent protein (GFP). HEK293 cells were plated on the slide for transfection and the fluorescence of the cells detected with a laser fluorescence scanner. A low magnification scan shows a regular pattern of fluorescent spots that matches the pattern in which we printed the GFP expression construct (Fig. 1b). A higher magnification image obtained through fluorescence microscopy shows that each spot is about 120–150 μm in diameter and consists of a cluster of 30–80 fluorescent cells (Fig. 1c). As in a conventional transfection, the total expression level in the clusters is proportional over a

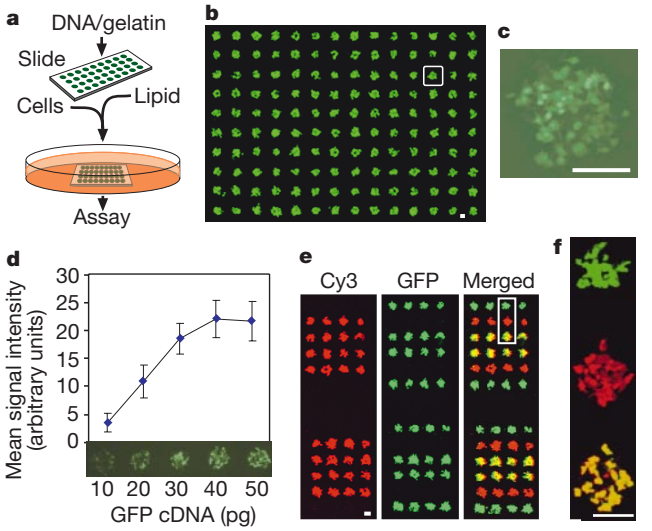

Figure 1 Well-less transfection of plasmid DNAs in defined areas of a lawn of mammalian cells. **a**, Protocol for making microarrays of transfected cells. **b**, Laser scan image of a GFP-expressing microarray made from a slide printed in a 14 × 10 pattern with a GFP expression construct. **c**, Higher magnification image obtained with fluorescence microscopy of the cell cluster boxed in **b**. Scale bar, 100 μm. **d**, Expression levels of cell clusters in a microarray are proportional, over a fourfold range, to the amount of plasmid DNA printed on the slide. Indicated amounts of the GFP construct assume a 1-nl printing volume. The graph shows the mean ± s.d. of the fluorescence intensities of the cell clusters ($n = 6$). The fluorescent image is from a representative experiment. **e**, Co-transfection is possible with transfected cell microarrays. Arrays with elements containing expression constructs for HA–GST, GFP or both were transfected and processed for immunofluorescence and imaged with a laser scanner. Cy3, cell clusters expressing HA–GST; GFP, cell clusters expressing GFP; merged, superimposition of Cy3 and GFP signals. Yellow colour indicates co-expression. Scale bar, 100 μm. **f**, Enlarged view of boxed area of scan image from **e**.

defined range to the amount of plasmid DNA used (Fig. 1d). As it is often useful to express two different plasmids in the same cells, we examined whether our technique is compatible with co-transfection. Arrays with elements containing expression constructs for GFP, haemagglutinin (HA)-tagged glutathione *S*-transferase (GST) or both were prepared and transfected. The cells growing on elements printed with both cDNAs express both encoded proteins, indicating that co-transfection does occur (Fig. 1e).

We first determined whether transfected cell microarrays could be used to identify gene products on the basis of their intrinsic properties. As a test case, we sought to use an array to identify the receptor for FK506, a clinically important immunosuppressant whose pharmacologically relevant target, FKBP12, is an intracellular protein[4–6]. We printed on a slide, in an easily recognizable pattern, elements containing expression constructs for Myc-tagged FKBP12, GFP or both. After the microarray of transfected cell clusters formed, we added radiolabelled FK506 to the tissue culture medium for 1 h before processing the slide for immunofluorescence and autoradiography. The radiolabelled FK506 bound to the array in a pattern of spots that exactly matched the pattern of cell clusters expressing FKBP12 (Fig. 2a). The GFP-expressing clusters and the non-transfected cells surrounding the clusters had background levels of signal (Fig. 2a). Detection of the bound FK506 with autoradiographical emulsion confirms, at the cellular level, colocalization between FKBP12 expression and FK506 binding (Fig. 2b). The clusters overexpressing FKBP12 had only a 3.1 ± 0.2-fold increase in the binding of radiolabelled FK506 compared with the GFP-expressing clusters (Fig. 2c), probably reflecting the high levels of endogenous FK506-binding proteins expressed in the cells[7]. Consistent with this, the prior addition of excess rapamycin, a competitive antagonist of FK506, not only eliminated the specific signal but also greatly lowered the background signal (Fig. 2c).

We also detected binding of a small molecule to a membrane protein expressed in specific clusters of a transfected cell microarray. We transfected slides printed with plasmids encoding an IRES–GFP cassette downstream of the cDNA for the dopamine D1 or serotonin 1A receptor. Cell clusters transfected with either plasmid expressed GFP, but a dopamine antagonist bound only to the cells transfected with the plasmid for the dopamine receptor (Fig. 2d).

We next used transfected cell microarrays to screen a collection, enriched for signalling molecules, of 192 epitope-tagged cDNAs cloned in expression vectors. All screens were performed in a blinded fashion without knowledge of the identity of the encoded proteins. We screened for gene products that increase the activity of kinase signalling pathways, induce apoptosis or are involved in cell–cell adhesion. After transfection of HEK293T cells on the array, we used phosphospecific antibodies to identify cell clusters with increased levels of phosphotyrosine or of the activated phosphorylated forms of several members of the mitogen-activated protein kinase (MAPK) family. Six cell clusters (1A2, 1C7, 1C9, 1C11, 1E3 and 1F6) had phosphotyrosine levels above background (Fig. 3a). The coordinates of the clusters match those of the wells of a microtitre plate containing the source cDNAs, and were used to ascertain the identity of the transfected cDNAs. This revealed that five of these clusters were expressing known tyrosine kinases (TrkC, Syk, Syn, Lck and Blk) whereas the sixth (1C9) encodes a protein of unknown function (Table 1). Screening of duplicate arrays for phospho-MAPKs showed that the clusters expressing Syk and Blk also had increased levels of activated phospho-JNK and phospho-p38 (Table 1). Syk is a known activator of these MAPKs[8], but this function has not been ascribed previously to Blk. Overexpression of Syn and Lck did not activate JNK or p38, indicating that, at least in HEK293 cells, not all cytoplasmic tyrosine kinases have this effect. To identify gene products that might have a function in apoptosis or cell adhesion, we examined the microarray for clusters containing cells with abnormal morphologies. We found one cluster (2E8) in which the cells were fragmented (Fig. 3b) and positive for the TdT-mediated dUTP nick end-labelling[9] reaction (data not shown),

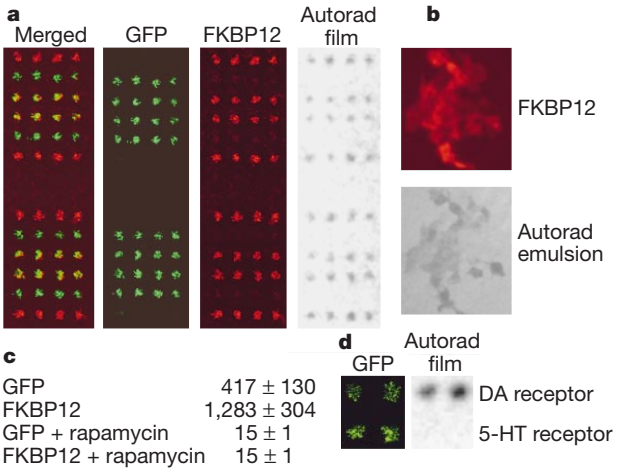

Figure 2 Detection of drug receptors on transfected cell microarray. **a**, Increased binding of tritiated FK506 to cell clusters expressing FKBP12. Laser scans show clusters expressing GFP, Myc-FKBP12 or both (merged). Film autoradiography detects binding of tritiated FK506 to the same array (autorad film). **b**, Colocalization between FKBP12-expressing cells and FK506-binding. Higher magnification image obtained by fluorescence microscopy of an FKBP12-expressing cluster (FKBP12). Emulsion autoradiography detects, with cellular resolution, binding of tritiated FK506 to the same cluster (autorad emulsion). **c**, Rapamycin competes with FK506 for binding to FKBP12-expressing clusters. Mean ± s.d. ($n = 10$) is shown in arbitrary units. **d**, Binding of tritiated SCH23390 to cell clusters expressing the dopamine D1 (DA) but not the serotonin 1A (5-HT) receptor (autorad film). Each receptor was expressed from a plasmid encoding a bicistronic messenger RNA containing an IRES–GFP element downstream of the receptor cDNA. Clusters on the left and right were transfected with 20 and 30 pg plasmid DNA, respectively. Fluorescence microscopy shows that all clusters express GFP.

Table 1 Phenotypes induced and subcellular distributions for cDNAs expressed in transfected cell microarray

Grid: coordinate	Phenotype/ distribution	Accession number	Gene name
1:A2	P-Tyr	U05012	TrkC
1:C7	P-Tyr, p-JNK, p-p38	L28824	Syk
1:C9	P-Tyr		Homologous to BT0590
1:C11	P-Tyr	M14333	Syn
1:E3	P-Tyr	M36881	Lck
1:F6	P-Tyr, p-JNK, p-p38	S76617	Blk
1:C2	Cytoskel. changes	M21616	β-PDGF receptor
2:E8	apoptosis	AF016266	TRAIL receptor 2
2:D10	Cell adhesion	X97229	NK receptor
2:F7	Cell adhesion	M98399	CD36
1:A9	Nuclear	U11791	Cyclin H
1:B5	Nuclear	M60527	Deoxycytidine kinase
1:B12	Nuclear	M60724	p70 S6 kinase kinase αI
1:C12	Nuclear	M90813	D-type cyclin
1:E4	Mitochondrial	U54645	Methylmalonyl-coA mutase
1:E10	Mitochondrial	J05401	Creatine kinase
1:G9	Nuc/cyto	U40989	Tat interactive protein (TIP60)
1:G10	Nuc/cyto	U09578	3pk kinase
2:A9	Nuclear	X83928	TFIID subunit TAFII28
2:A12	Nuc/cyto	M62831	ETR101
2:B6	Nuc/cyto	X06948	IgE receptor α-subunit
2:B12	Nuclear	X63469	TFIIE β-subunit
2:C5	Nuclear	M76766	General transcription factor IIB
2:C7	Nuc/cyto	M15059	CD23A
2:C12	Nuclear	X80910	PP1, β-catalytic subunit
2:D4	Nuclear	AF017307	Ets-related transcription factor
2:E7	Nuclear	X63468	TFIIE-α
2:E12	Nuclear	U22662	Orphan receptor LXR-α
2:F8	Nuclear	L08895	MEF2C
2:F12	Nuclear	AF028008	SP1-like transcription factor
2:G2	Nuc/cyto	U37352	PP2A, regulatory B′α1 subunit

Nuc/cyto, nuclear and cytoplasmic staining was visible. P-Tyr, p-JNK1 and p-p38 indicates that cluster had staining of phosphotyrosine, phospho-JNK and phospho-p38, respectively, above background.

which is consistent with their expression of the TRAIL receptor 2, an apoptosis-inducing protein[10]. In another cluster (2F7), the plasma membranes of adjoining cells were in close contact to each other (Fig. 3b). This cluster expressed CD36, a cell-surface protein with functions in cell–cell recognition and adhesion[11]. Other cell phenotypes that are consistent with the potential functions of the cDNAs expressed in the clusters were also present on the microarray (Table 1). Thus, screens using simple detection methods (phosphospecific antibodies and visual inspection) revealed diverse cellular phenotypes in the transfected cell microarray and identified both known and new roles for proteins.

The subcellular distribution of a protein can provide crucial insights to its cellular functions, and its determination is an important part of characterizing a gene of unknown function. Therefore, we also examined, through visual inspection, the subcellular distribution of the proteins expressed in the cell microarrays made with the cDNA collection. We identified 22 clusters expressing proteins with distinct, non-cytoplasmic subcellular distributions (Table 1). Several proteins that are known transcription factors, including ETR101, MEF2C and SP1-like transcription factor, were mainly located in the cell nucleus. This was also true for other proteins with potential nuclear roles, such as deoxycytidine kinase, 3pK (chromosome 3p kinase) and TIP60, as well as others, like phosphatase 1-β (PP1β), whose subcellular distribution has not been ascertained previously. The location of PP1β in the nucleus was unexpected and differs from that of its close homologue, PP1γ, which is also expressed on the array but was found exclusively in the cytoplasm (data not shown).

Transfected cell microarrays have distinct advantages over conventional expression cloning strategies using fluorescence-activated cell sorting or sib selection[12]. First, cDNAs do not need to be isolated from the cells showing the phenotype of interest. This significantly accelerates the pace of expression cloning and allows for screens using a variety of detection methods (including those incompatible with cell viability) such as autoradiography and *in situ* hybridization. Our experiments took days to perform instead of the weeks or months necessary with other expression cloning strategies. Second, as the signal is concentrated in a well defined small area in a transfected cell microarray, cDNAs can be identified whose expression produces only small increases in signal over background. This capacity is well demonstrated by our detection of the receptor for FK506 in host cells with high levels of endogenous FK506-binding proteins. The relatively small increase in binding of the drug over background caused by the overexpression of FKBP12 would have been difficult to detect in the early rounds of sib selection. Third, transfected cell microarrays can be used to screen live cells, allowing the detection of transient phenotypes, such as a change in the intracellular calcium concentration or the movement of a GFP-tagged protein. Fourth, being compact and easy to handle, transfected cell microarrays have economies of scale. Because the microarrays are printed with the same robotic arrayers as traditional DNA arrays, it is feasible to achieve densities of up to 6,000–10,000 cell clusters per standard slide. At these densities, the entire set of human genes could be expressed on a small number of slides. The main difficulty in creating such pan-genomic arrays is the work required in making the cDNA expression constructs to print on the slides. This disadvantage is shared by other microarray-based technologies that use defined sets of cDNAs. Despite the effort required, projects to make collections of full-length cDNAs for all human genes are underway[13–16] (see also www.hip.harvard.edu/research.html) and will be valuable tools for making the arrays we describe.

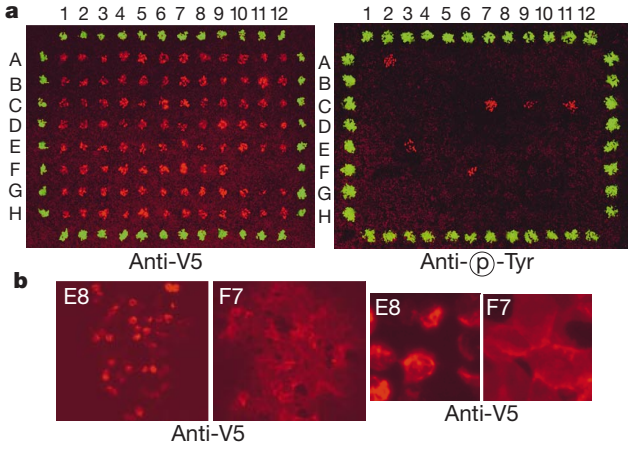

Figure 3 Identification of gene products inducing cellular phenotypes of interest. **a**, Detecting components of tyrosine kinase signalling cascades. V5-epitope-tagged cDNAs (192) in expression vectors were printed in two 8 × 12 subgrids named grid 1 and 2. For ease of determining the coordinates of cell clusters within the arrays, a border around each array was printed with a GFP expression construct. After transfection, separate slides were processed for anti-V5 or anti-phosphotyrosine (anti-p-Tyr) immunofluorescence and Cy3 and GFP fluorescence was detected. Merged images of grid 1 show location of clusters expressing V5-tagged proteins (left panel) and having increased level of phosphotyrosine (right panel). In this particular array, no DNA was printed at coordinates F10–12. **b**, Two examples of the morphological phenotypes detectable in the transfected cell microarrays described in **a**. Clusters shown are E8 and F7 from grid 2.

We have described arrays in which the transfected plasmids direct gene overexpression. However, as antisense technology improves or other methods emerge for decreasing gene function in mammalian cells, it may be possible to also use transfected cell microarrays to screen for phenotypes caused by loss of gene function. The key to spatially restricting transfection without wells is the immobilization of the plasmid DNA in a gel from which it is only accessible to nearby cells. This concept may be generally applicable to the high-throughput and well-less screening in mammalian cells of other molecules, such as chemical compounds, proteins or oligonucleotides. □

Methods

Plasmids

EGFP (enhanced GFP) was expressed from the retroviral vector pBABEpuro[20], HA–GST and Myc-tagged FKBP12 from the cauliflower mosaic virus (CMV)-driven prk5 vector[21], and the human dopamine D1 and serotonin 1A receptors from the LTR (long terminal repeat) driven, bicistronic pMSCV–IRES–EGFP vector. The Genestorm cDNAs are cloned into the CMV-driven pcDNA3.1/GS vector (Invitrogen). These clones were picked at random by Invitrogen from their collection of cDNAs and the clone names are provided in the Supplementary Information. DNA was purified with the Plasmid Maxi or QIAprep 96 Turbo Miniprep kits (Qiagen), and always had an A260/A280 greater than 1.7.

Microarray printing

A robotic arrayer (PixSys 5500; Cartesian Technologies) equipped with stealth pins (ArrayIt SMP4; Telechem) was used to print a plasmid DNA/gelatin solution contained in a 384-well plate onto CMT GAPS glass slides (2549, Corning). The pins deposited about 1-nl volumes 400 μm apart using a 25–50-ms pin-down-slide time in a 55% relative humidity environment. Printed slides can be stored at 4 °C or at room temperature in a vacuum dessicator. Storage for long periods of time (>3 months) has not appreciably affected performance. Preparation of aqueous gelatin solution is important and is as follows. 0.2% gelatin (w/v) (G-9391; Sigma) was dissolved in MilliQ water by heating and gentle swirling in a 60 °C water bath for 15 min. The solution was cooled slowly to room

temperature and filtered through a 0.45-μm cellular acetate membrane and stored at 4 °C. We diluted concentrated solutions of DNA in the gelatin solution to keep the gelatin concentration >0.17%. Unless otherwise specified, final plasmid DNA concentrations were 0.033 μg μl^{-1}. For the Genestorm clones, the final DNA concentration was 0.040 μg μl^{-1}.

Reverse transfection of microarrays

We used the Effectene transfection kit (301425; Qiagen) as follows. In a 1.5-ml microcentrifuge tube, 16 μl enhancer was added to 150 μl EC buffer, mixed, and pre-incubated for 5 min at room temperature. 25 μl Effectene lipid was added, mixed and the entire volume pipetted onto a 40 × 20-mm cover well (PC200; Grace Bio-Labs). A slide with the printed side down was placed on the cover well such that the solution covered the entire arrayed area while also creating an airtight seal. After a 10–20-min incubation, the cover well was prised off the slide with forceps and the transfection reagent removed carefully by vacuum aspiration. The slide was placed printed side up in a 100 × 100 × 10 mm square tissue culture dish and 1 × 10^7 actively growing HEK293T cells in 25 ml medium (DMEM with 10% IFCS (inactivated fetal calf serum), 50 units ml^{-1} penicillin and 50 μg ml^{-1} streptomycin) were poured into the dish. Three slides can be transfected side by side in this fashion. The cells grew on the slide for 40 h before fixing for 20 min at room temperature in 3.7% paraformaldehyde/4.0% sucrose in PBS. We have also tested other commonly used mammalian cells lines, such as HeLa and A549 cells, and obtained similar results but with transfection efficiencies of 30–50% of those obtained with HEK293T cells.

Immunofluorescence

For immunofluorescence staining, the cells were fixed as above, permeabilized in 0.1% Triton X-100 in PBS for 15 min and probed with primary and secondary antibodies as described[21]. Primary mouse monoclonal antibodies (500 μl per slide) were used at the following concentrations: 1:500 anti-HA ascites fluid (BaBCo), 2 μg ml^{-1} anti-Myc 9E-10 (Calbiochem), 2 μg ml^{-1} anti-V5 (Invitrogen), and 10 μg ml^{-1} 4G10 anti-phosphotyrosine (Upstate Biotechnologies). Primary rabbit polyclonal antibodies (500 μl per slide) were used at the following concentrations: 0.7 μg ml^{-1} anti-p38 pTGpY (Promega), 0.7 μg ml^{-1} anti-JNK pTPpY (Promega), and 0.5 μg ml^{-1} anti-ERK1/2pTEpY (NEB). Secondary antibodies were Cy3-labelled anti-mouse or anti-rabbit antibodies produced in donkeys (Jackson Immunoresearch) and were used at 3.1 μg ml^{-1}.

Laser scanning and fluorescence microscopy

We imaged microarrays at a resolution of 5 μm with a laser fluorescence scanner (ScanArray 5000; GSI Lumonics). GFP and Cy3 emission was measured separately after sequential excitation of the two fluorophores. To obtain images at cellular resolution, cells were photographed with a conventional fluorescence microscope. To measure fluorescence intensity, cell clusters were photographed and the signal intensity quantified with Image Quant (Fuji). All images were pseudocoloured and superimposed using Photoshop 5.5 (Adobe Systems).

FK506 and SCH23390 autoradiography

We added 5 nM dihydro-FK506 [propyl-^{3}H] (NEN) to the cell medium containing the microarrays for 1 h before rinsing once with PBS at room temperature and fixation. When used, rapamycin was added at 1 μM for 30 min before FK506 addition. Before autoradiography, slides were processed for anti-Myc immunofluorescence, scanned at 5-μm resolution and photographed using a fluorescence microscope. We then exposed slides to tritium-sensitive film (Hyperfilm, Amersham) for 4 days. Autoradiographical emulsion was performed as described by the manufacturer (EM-1; Amersham). Image Quant (Fuji) was used to quantify 0.01-mm^2 areas of autoradiograms. For the dopamine receptor experiments, 1 nM SCH23390 [*N*-methyl-^{3}H] (NEN) was added to the cell medium containing the microarrays for 1 h before rinsing once with PBS at room temperature and fixation. Before processing for autoradiography as above, we photographed GFP fluorescence using a fluorescence microscope.

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An aminoacyl tRNA synthetase whose sequence fits into neither of the two known classes

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Aminoacyl transfer RNA synthetases catalyse the first step of protein synthesis and establish the rules of the genetic code through the aminoacylation of tRNAs. There is a distinct synthetase for each of the 20 amino acids and throughout evolution these enzymes have been divided into two classes of ten enzymes each[1,2]. These classes are defined by the distinct architectures of their active sites, which are associated with specific and universal sequence motifs[1–5]. Because the synthesis of aminoacyl-tRNAs containing each of the twenty amino acids is a universally conserved, essential reaction, the absence of a recognizable gene for cysteinyl tRNA synthetase in the genomes of Archae such as *Methanococcus jannaschii* and *Methanobacterium thermoautotrophicum*[6–8] has been difficult to interpret. Here we describe a different cysteinyl-tRNA synthetase from *M. jannaschii* and